REMARKS

Applicants reassert their arguments that the application is in condition for allowance. If the PTO maintains its rejection of the application after considering the remarks below, applicants request entry of the Amendment to the claims in order to place the application in condition for allowance. No new matter enters by the amendment adding new claims and canceling the old claims. If the Amendment is entered, claims 52-75 will be pending.

The new claims specifically recite adenoviral vectors or adenoviruses. Applicants disagree with the PTO's comments on the type of vector that one skilled in the art could have used, as explained below. Furthermore, applicants disagree with the PTO's comments concerning the use of the vector or expression system language, for example. However, in order to further the prosecution of this case, applicants propose new claims coinciding with the PTO's conclusions that "a method for treating ALS comprising systemic administration of a pharmaceutical composition comprising an adenoviral vector encoding a neurotrophic factor and a pharmaceutical composition comprising two adenoviral vectors encoding two neurotrophic factors" are enabled (*see* page 2 of Paper No. 14). The new claims also incorporate language that substitutes for the "expression system," "nucleic acids," and "construct" terms (*see* pages 3-4 of Paper No. 14) with equivalent terms.

This application is in condition for allowance whether or not the Amendment is entered. Applicants in no way disclaim any subject matter or concede that any subject matter is unpatentable by the Amendment.

Rejection under 35 U.S.C. § 112, First Paragraph

Claims 26-35, 37-42, 44, 45, 49, 50, and 51 stand rejected under 35 U.S.C. § 112, first paragraph, as the specification allegedly fails to enable one of skill in the art to make and use the claimed invention. Applicants respectfully disagree.

By way of a brief summary, the PTO asserts that while adenoviral vectors are enabled, other vectors are not (*see* pages 2-3 of Paper No. 14).

Applicants note that the specification includes several examples showing how to make and use the compositions, expression systems, expression cassettes, and vectors of the disclosed

invention. That recombinant adenoviral vectors are shown in Examples 5-10 does not in any way indicate that other compositions comprising expression cassettes cannot be selected and used. In fact, Example 2.1 clearly shows that plasmid vectors, for example, can be selected. Thus, applicants have clearly stated that the application enables the invention encompassing at least plasmid vectors in addition to adenoviral vectors. It is therefore the PTO's burden to establish that applicants' presumptively enabling specification does not enable the claims. *In re Marzocchi*, 169 U.S.P.Q. 367 (CCPA 1971).

With respect to selecting vectors in particular, one skilled in the art was familiar with many different ways to use an expression system or cassette in a gene transfer technique. Examples from different methods involving, for example, plasmids, retroviruses, adeno-associated viruses, liposomes, and eletrotransfer techniques to name a few, could have been selected. As mentioned previously, the WO 99/01175 document shows how one of skill in the art could have used techniques to improve gene delivery in a method that would seem very relevant to treating ALS (*see* Response filed October 23, 2001).

In addition, the WO 95/25804 document (of record), mentioned in the specification at Example 2.1, page 25, discusses plasmid vectors that can be used. An excerpt from pages 13-14 of this document appears below.

Exemple 3. Fonctionalité des vecteurs pSh-Ad-BDNF et pSh-Ad-BDNFtag

La capacité des vecteurs pSh-Ad-BDNF et pSh-Ad-BDNFtag à exprimer sur culture cellulaire une forme biologiquement active du BDNF a été démontrée par transfection transitoire de cellules COS1. Pour cela, les cellules (2.10^6 cellules par

boite de 10 cm de diamètre) ont été transfectées (8 µg de vecteur) en présence de Transfectam. Après 48 heures, le surnageant de culture des cellules a été récolté. Des dilutions sérielles (1/200 et 1/50) de ce surnageant ont ensuite été ajoutées à des cultures primaires de neurones du septum (Hefti et al. In Dissection and Tissue cultures : Manual of the Nervous System (1989) 172, Alan R. Liss, Inc). L'effet trophique (survie cellulaire et pousse neuritique) sur ces cultures a été observé après coloration, et l'effet différentiateur par dosage de l'expression de l'enzyme choline acétyl transférase (ChAT), selon la technique décrite par Fonnum (J. Neurochem. 24 (1975) 407).

This excerpt can be roughly translated as:

> The capacity of vectors pSh-Ad-BDNF and pSh-Ad-DBDNFtag to express a biologically active form of BDNF in a cell culture was demonstrated by transitory transfection of COS1 cells. For this experiment, the cells ($2x10^6$ cells per plate of 10 cm diameter) were transfected (8 ug vector) in the presence of Transfectam. After 48 hours, the cell culture supernatant was collected. Serial dilutions (1/200 and 1/50) of the supernatant were then added to primary cultures of neurons from the septum (Hefti et al., In: Discussion and Tissue cultures: Manual of the Nervous System (1989) 172, Alan R. Liss, Inc.). The trophic effect (cell survival and neurite growth) on the cultures was observed after staining, and the differentiation effect was determined by assaying for the expression of the enzyme choline acetyl transferase (ChAT), which technique is described by Fonnum, J. (Neurochem. 24: 407 (1975)).

Clearly, an adequate description of plasmid vectors, *inter alia,* exists in this excerpt. Applicants submit that the information explicitly or implicitly included in the applicants' specification through the reference to WO 95/25804 indicates that, armed with the applicants' disclosure on compositions and methods to treat ALS, one of skill in the art could have used the specifically disclosed plasmid vectors, for example, in addition to the adenovirus vectors.

As further evidence, applicants enclose a copy of WO 00/18933. This document indicates that, with the knowledge that applicants provide in their specification, one of skill in the art can select and use an expression cassette or construct in an adenoviral vector or a plasmid. The WO 00/18933 document shows both types of vectors in delivering the same genes in a human gene transfer experiment.

Thus, plasmids, naked nucleic acids, or adenoviral vectors, for example, could be used to introduce the expression system or expression cassette of the disclosed invention. Since applicants have already demonstrated how to make and use the adenoviral vectors, as the PTO agrees, there would seem to be no reason to questions one's ability to select a desirable vector for delivering the expression system encompassing a plasmid, for example.

Applicants respectfully submit that there is no reason under the appropriate laws to require any additional showing that the claimed invention of claims 26-35, 37-42, 44, 45, 49, 50, and 51 can be used. The Examples and specification demonstrate enabling uses.

Applicants respectfully request withdrawal of this rejection.

Rejection under 35 U.S.C. § 112, Second Paragraph

Claims 26-51 stand rejected under 35 U.S.C. § 112, second paragraph, as the claims are allegedly indefinite. Applicants respectfully disagree.

At pages 3-4 of Paper No. 14, the PTO asserts that the terms or phrases "expression system," "construct," and "two nucleic acids" are indefinite. Applicants have already explained why one of skill in the art would consider an expression system, for example, an understandable term. Applicants hereby incorporate by reference the comments of the Response filed October 23, 2001, at pages 4-5. However, the new claims remove or replace the terms or phrases noted above without changing the scope of the subject matter claimed.

Applicants respectfully request withdrawal of this rejection.

CONCLUSION

Applicants maintain that this application is in condition for allowance. If the Examiner believes that an interview with applicants' representative, either by telephone or in person, would further prosecution of this application, we would welcome the opportunity for an interview.

Applicants have provided for an extension of time above. No additional extension of time fees, requests for extension of time, or other petitions, additional claim fees, or other fees are believed to be necessary to enter and consider this paper. If, however, any extensions of time are required or any fees are due in order to enter or consider this paper or enter or consider any paper accompanying this paper, including fees for net addition of claims, applicants hereby request any extensions or petitions necessary and the Commissioner is hereby authorized to charge our Deposit Account No. 50-1129 for any fees. If there is any variance between the fee submitted and any fee required, or if the payment or fee payment information has been misplaced

or is somehow insufficient to provide payment, the Commissioner is hereby authorized to charge or credit Deposit Account No. 50-1129.

Respectfully submitted,
WILEY REIN & FIELDING LLP

Dated: May 31, 2002

By: _____
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(54) Title: IMMUNOTHERAPY OF CANCER THROUGH EXPRESSION OF TRUNCATED TUMOR OR TUMOR-ASSOCIATED ANTIGEN

(57) Abstract

DNA constructs for truncated forms of cancer-specific or cancer associated antigens are included in plasmid or viral expression vectors. The rationale to use constructs for truncated and not for full-size molecules is to eliminate side effects (toxicity, signal transduction, etc.) arising from expressed proteins and/or, in cases where such molecules are expressed on the membrane, secreted, or released in the extracellular environment, to prevent formation of antibodies against them. The extracellular portion of the human prostate specific membrane specific antigen (XC-PSMA) has been cloned. Patients were treated either by injection of DNA coding for XC-PSMA in a mammalian expression vector under the CMV promoter or/and by a replication-defective adenoviral vector (Ad5)hat contains an expression cassette for the XC-PSMA. In a third method dendritic cells are isolated from a patient and are treated by exposure to the plasmid or adenovirus used in the previous two treatments. The dendritic cells are then injected into the patient. In some patients, the progression of metastatic prostate cancer is retarded or stopped.

TITLE OF THE INVENTION:

IMMUNOTHERAPY OF CANCER THROUGH EXPRESSION OF TRUNCATED TUMOR- OR TUMOR-ASSOCIATED ANTIGEN

CROSS-REFERENCE TO RELATED APPLICATIONS.

Not Applicable.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT.

Not Applicable.

BACKGROUND OF THE INVENTION

(1) FIELD OF THE INVENTION

The present invention relates to compositions and methods for immunotherapy of human cancer patients.

(2) DESCRIPTION OF RELATED ART INCLUDING INFORMATION DISCLOSED UNDER 37 CFR 1.97 AND 37 CFR 1.98.

All normal human nucleated cells express on their membrane small protein fragments derived from de novo protein synthesis. These so-called peptides are associated with the major histocompatibility complex (MHC) class I molecules and form the antigens which are recognized by CD8 cytotoxic T-lymphocytes (CTLs). Such recognition is important for the elimination of virally infected cells, of tumor cells, or of cells that contain intracellular parasites. For this to occur potentially antigen-reactive T cells need to be "pre-educated" by recognizing the antigen in question on the membrane of professional antigen -presenting cells (dendritic cells) (APCs, DCs) which, in addition to the antigen, provide co-stimulatory "maturation" signals to the T cells. In the absence of such signals the T cells become paralyzed and tolerant to the antigens in question.

Tumor cells, which are not professional APCs, do not stimulate CTL generation and are not rejected by the immune system. For the generation of an immune response against a tumor the tumor antigen(s) need(s) to be expressed by professional APCs. This presentation has been accomplished by in vitro exposure of dendritic cells to tumor lysates that presumably contain tumor antigens, to purified tumor antigens or, to peptides derived from such antigens.

Another possibility to achieve expression of antigen-derived peptides is by introducing into dendritic cell desoxy- (DNA) or ribonucleic acid (RNA) that encodes the antigen of interest. Cells transfected with the plasmid DNA transiently synthesize the protein and the peptides that are obtained during the synthesis are then expressed in association with MHC. For example, patient's cells grown in vitro are transfected with plasmids, containing the DNA, or with the RNA of interest or infected with a recombinant viral vector that contains the DNA or RNA, and then returned to the patient. Another possibility is to directly immunize the patient with the plasmid ("nude" DNA immunization) or with the recombinant viral vector.

A major problem with this technique comes from the possible adverse effects of the expressed products on the patient's health or on cell viability. Since the function of these tumor-associated or tissue-specific antigens is largely unknown, their synthesis and release by patient's cells in vivo may lead to serious side effects. Furthermore, in cases where dendritic cells are transfected in vitro, expression of a functional protein may alter dendritic cell viability, change their migration pattern or their ability to provide co-stimulation to T cells.

The present invention discloses the idea for the introduction of specific changes in the DNA or RNA encoding the antigen in question as a way of solving this problem. Such changes result in the expression of functionally inactive products without affecting the efficiency of transcription and translation of the DNA, the translation of the RNA, or the generation of antigenic peptides. Specifically the present invention discloses the development of a DNA, which leads to expression of a truncated form of the human prostate specific membrane antigen (PSMA). In particular, we have developed a DNA construct with deletions of the membrane and the intracellular portions of the human PSMA. The resulting DNA encoding the extracellular portion of the PSMA (XC-PSMA) has been incorporated in mammalian expression vectors. PSMA is a type II protein, it lacks a hydrophobic signal sequence and therefore is not secreted by the cell that produces it. Since our construct lacks membrane and cytoplasmic sequences, the resulting protein is not expressed on the membrane, therefore does not transduce signals and is not released from the membrane. Cells transfected with the XC-PSMA plasmid retain viability and express PSMA-derived peptides.

Furthermore, since the synthesized protein is not released but remains confined to the intracellular milieu, there is no production of antibodies directed against the protein and the immune response remains strictly cell-mediated. The exquisite engagement of cell-mediated immunity against a particular antigen is very important especially in cases where the target antigen of interest is expressed on normal tissues that are anatomically sequestered in immuno-privileged sites such as the eye, brain, testis etc. Those tissues are inaccessible to cell mediated injury, but readily damaged by antibodies. Immunotherapy based on eliciting cellular responses to differentiation (tyrosinase; gp100; TRP1; TRP2; MART-1/Melan-A; membrane-associated mucin, MUC-1 mucin) or normal tissue-specific (PSMA, PSA) antigens constitute an example where the production of antibodies against the target must not occur.

In the first method of treating of prostate cancer patients, the plasmid is injected intradermally. In a second method of treatment, the plasmid is incorporated into the genome of a replication-deficient adenovirus, which is injected intradermally into a patient. In a third method of treatment, CD14+ monocyte cells of a prostate cancer patient are isolated and matured into dendritic cells (DC) and transfected with either the plasmid or the adenovirus of the first two methods. The DC are then stimulated to express MHCs and are infused back into the prostate cancer patient where they stimulate autologous T-cells. These stimulated T-cells then destroy both normal and malignant prostate

cells.

The effect of all of these treatments is to either by-pass the normal tolerance for self-antigens or the tolerance to tumor antigens. This will enable the cytolysis of target normal and malignant prostate cells normally shielded from immune recognition. The destruction of normal prostate cells by this procedure is not detrimental to the patient. A malignant prostate (with its mixture or normal and malignant cells) customarily is destroyed through surgery or radiation in the conventional primary treatment for this disease.

U.S. Patent 5,227,471 discloses the structure of the prostate-specific membrane antigen. A method for treating prostate cancer was disclosed which involves an antibody directed against the prostate-specific membrane antigen and a cytotoxic agent conjugated thereto. However, since the PSMA is expressed on normal brain cells, use of antibodies which can transverse through the blood-brain barrier and damage normal brain cells is not acceptable. Methods for imaging prostate cancer and an immunoassay for measuring the amount of prostate-specific membrane antigen also were disclosed.

U.S. Patent 5,788,963 discloses the use of human dendritic cells to activate T cells for immuno-therapeutic response against primary and metastatic prostate cancer. Human dendritic cells are isolated and exposed to PSMA or peptides derived thereof in vitro. The PSMA or peptides are believed to exchange with peptides already bound to MHC molecules on the dendritic cells and thereby to be expressed in an imunogenic manner, enabling the DC to stimulate killer cells which then lyse prostate cells.

U.S. Patents 5,227,471 and 5,788,963 are incorporated by reference herein.

The present invention differs from the prior art in that it causes the DC to present an antigen derived from prostate cancer cells on their surface through transfection with a plasmid or adenovirus. The transfection may occur in vivo using injected plasmid or adenovirus. Alternatively, the transfection may occur in vitro using purified DC precursor cells isolated from the prostate cancer patient's blood. If transfection is done in vitro, the transformed cells are injected into the patient. Transfected DC cells are superior to DC cells, which have been exposed to antigen in vitro because both their loading with antigen-derived peptide and their ability to stimulate killer cells are more efficient. In addition, in vivo transfection using a plasmid or adenovirus is less laborious and less expensive than in vitro methods. The use of transfected cells avoids the necessity of identifying peptides capable of binding to different HLA phenotypes, as is required in methods, which involve the addition of peptides to cells. Finally, the use of a DNA sequence that encodes a truncated molecule of the PSMA guarantees that the protein is not released by the transfected cells and no antibodies against the target protein that are potentially hazardous to normal brain tissue are produced. The methods of the present invention bypass the normal tolerance for self-antigens. This enables the cytolysis of target cells normally shielded from immune recognition.

Another application involves treatment of melanoma patients. Melanocyte differentiation antigen

MART-1 is a common melanoma antigen recognized by many CTLs from melanoma patients. It represents a membrane protein of 118 aminoacids and a single transmembrane domain. Either DNA encoding for a truncated form with no transmembrane domain or a full-size protein with no leading sequence is included in a plasmid or viral expression vector and used for immunotherapy similar to the one described for prostate cancer patients.

Another application involves treatment of breast, ovary, uterine, prostate or lung cancer patients. Her-2/neu antigen is a member of the epidermal factor receptor family and is presumed to function as a growth receptor. It is a transmembrane protein and is expressed during fetal development and very weekly on normal cells as a single copy. Amplification of the gene and/or overexpression of the associated protein have been identified in many human cancers such as breast, ovary, uterus, stomach, prostate and lung. DNA encoding for a truncated form of the Her-2/neu protein lacking the transmembrane portion and the leading sequence is constructed and included in a plasmid or viral vector(s) and used for in vitro or in vivo modification of patient dendritic cells and for immunotherapy.

DETAILED DESCRIPTION OF THE INVENTION.

The present invention discloses the idea for the construction of genetically modified forms of polynucleotides encoding either tissue-specific or tumor antigens and for the use of such constructs for immunotherapy of primary or metastatic cancer. The genetic modification of the constructs leads to expression of either functionally inactive products or prevents functionally active molecules from being secreted or expressed on the membrane of transfected cells. Such genetic modifications, however, do not affect the antigenicity of the expressed protein, its primary structure or the generation of peptides available for binding to cell's MHC molecules. The polynucleotide may be either a DNA or RNA sequence. When the polynucleotide is DNA, it can also be a DNA sequence, which is itself non-replicating, but is inserted into a plasmid, and the plasmid further comprises a replicator. The DNA may be a sequence engineered so as not to integrate into the host cell genome. The polynucleotide sequences may code for a polypeptide which is either contained within the cells or secreted therefrom, or may comprise a sequence which directs the secretion of the peptide.

The DNA sequence may also include a promoter sequence. In one preferred embodiment, the DNA sequence includes a cell-specific promoter that permits substantial transcription of the DNA only in predetermined cells. The DNA may also code for a polymerase for transcribing the DNA, and may comprise recognition sites for the polymerase and the injectable preparation may include an initial quantity of the polymerase.

In many instances, it is preferred that the polynucleotide is translated for a limited period of time so that the polypeptide delivery is transitory. The polypeptide may advantageously be a therapeutic polypeptide, and may comprise an enzyme, a hormone, a lymphokine, a receptor, particularly a cell surface receptor, a regulatory protein, such as a growth factor or other regulatory agent, or any other

protein or peptide that one desires to deliver to a cell in a living vertebrate and for which corresponding DNA or mRNA can be obtained.

In preferred embodiments, the polynucleotide is introduced into muscle tissue; in other embodiments the polynucleotide is incorporated into tissues of skin, brain, lung, liver, spleen or blood. The preparation is injected into the vertebrate by a variety of routes, which may be intradermally, subdermally, intrathecally, or intravenously, or it may be placed within cavities of the body. In a preferred embodiment, the polynucleotide is injected intramuscularly. In still other embodiments, the preparation comprising the polynucleotide is impressed into the skin. Transdermal administration is also contemplated, as is inhalation.

One example of this approach is the use of a DNA that encodes a truncated form of the human PSMA, which lacks the membrane, and cytoplasmic portions of the molecule. Such DNA has been included by us into mammalian expression vectors: a plasmid and a propagation deficient virus.

For treatment of prostate cancer patients, dendritic cells are prepared by transfection using either a plasmid or a recombinant replication-deficient adenovirus whose DNA includes DNA encoding a truncated fragment of the prostate specific membrane antigen. Dendritic cells may be transfected in vivo by injection of plasmid or recombinant replication-deficient adenovirus in the patient. Alternatively the DC may be transfected (infected) in vitro by treating isolated dendritic cell precursor cells with plasmid (or recombinant replication-deficient adenovirus). The dendritic cells are then injected into the patient.

Without wishing to be held to this theory, it is the inventors' belief that successful immunotherapy requires that the target antigen be presented by a DC simultaneously to both the helper (CD4+ T cells) and the effector (CD8+ T cells) arms of the immune system. Recognition by CD4+ T cells requires that antigenic peptides be expressed in conjunction with class II MHC molecules on the DC surface. This can be achieved by in vivo or in vitro transfection of DC with plasmid or infection of DC with recombinant adenovirus, both of which carry the DNA for the extracellular fragment of PSMA.

PSMA expression is restricted to prostate epithelial cells (Horoszewicz JS, Kawinski E and Murphy GP. Monoclonal antibodies to a new antigenic marker in epithelial prostate cells and serum of prostate cancer patients. Anticancer Res. 7:927;1987) and human brain tissue (Luthi-Carter R, Barczak AK, Speno H, Coyle JT. Molecular characterization of human brain N-acetylated alpha-linked acidic dipeptidase (NAALADase). J Pharmacol. Exp. Therap. 286:1020;1998). The antigen is expressed on normal and neoplastic prostate cells in the prostate or in prostate tumor metastases. While other marker antigens for prostate carcinoma such as prostate acid phosphatase and the prostate specific antigen (PSA) are secreted antigens, PSMA is an integral membrane glycoprotein.

Cloning of extracellular fragment of PSMA

cDNA of PSMA extracellular fragment (2118 bp) was obtained using total mRNA from the

prostate tumor cell line LNCaP.FGC - CRL 1740 (ATCC). A PSMA-specific 3'-primer was used for reverse transcription of mRNA which was performed using RT from avian myeloblastosis virus (Boehringer). Th resulting cDNA was then amplified using High Fidelity PCR System (Boehringer), and the gel purified PCR product of expected length was cloned into pCR2.1 vector (Invitrogen). Two clones were selected and checked by DNA sequencing. The resulting construct contains a free of mutation extracellular portion of PSMA with NotI-Kozak sequence introduced by PCR at its 5' end and SfuI site at its 3' end.

Preparation of the mammalian expression vector for subcloning of the extracellular portion of PSMA.

The modified cloning vector pcDNA3.1 (Invitrogen) was used for subcloning. The vector provides human cytomegalovirus (CMV) immediate-early promoter/enhancer region permitting efficient, high-level expression of recombinant protein as well as 3' flanking region containing bovine growth hormone polyadenilation signal for efficient transcription termination and for increasing the half life of the mRNA in vivo. The neomycin resistance gene (NRG) was removed by digestion with NaeI endonuclease and ligation of the NRG-free fragment of the plasmid following gel purification.

Subcloning of the extracellular portion of PSMA into a mammalian expression vector.

The extracellular fragment of the PSMA was sub-cloned into a modified mammalian expression vector pcDNA3.1 by NotI-SfuI cloning sites. Both NotI and SfuI sites as well as Kozak sequence were introduced during the RT-PCR step of the cloning.

Deposit of modified mammalian expression vector pcDNA3.1.

The modified mammalian expression vector was deposited as Designation Number 203168 on August 28, 1998 at the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia 20110.

Preparation of a replication-defective recombinant adenovirus Ad5-PSMA.

Ad5-PSMA recombinant adenovirus was prepared using the kit available from Quantum Biotechnology Inc. The transfer vector was constructed by subcloning of the extracellular PSMA fragment into the plasmid pAdBN (Quantum). For this purpose the PSMA fragment was initially sub-cloned into an unmodified pCDNA3.1 vector (Invitrogen). The portion of the plasmid that contains the CMV promoter-PSMA fragment-PolyA signal was cut using BglII and SmaI restriction endonucleases. The resulting product was purified on an agarose gel and subcloned by BglII-EcoRV cloning sites into pAdBN transfer vector (Quantum Biotechnologies Inc., Montreal, Canada).

The transfer vector was linearized with ClaI and co-transfected with linearized Adenovirus DNA in 293A cells. The recombinant adenovirus was purified three times and clones that were positive for PSMA expression were selected by immunoblotting. The positive clone was amplified in 293 cells and then purified on two successive CsCl gradients. Finally the purified virus was dialyzed against PBS-5%

sucrose.

The replication-defective recombinant adenovirus Ad5-PSMA. was deposited as Designation Number _____ on August 28, 1998 at the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia 20110.

In vitro experiments:

Gene Transfer Using Replication-Deficient Adenovirus.

Peripheral blood mononuclear cells (PBMC) from healthy anonymous donors were isolated from freshly drawn blood by density centrifugation on Ficol-Paque at 468 g at 22°C for 30 minutes. PBMC were resuspended in RPMI with 5% autologous serum (complete medium) culture medium at 1 X 10 6 cells/ml and allowed to adhere onto 175 cm^2 polystyrene tissue culture flask. The flasks are incubated at 37°C and shaken every 20 minutes during incubation. After 1 hrs at 37 C, non-adherent cells are removed and adherent cells are cultured in 30 ml medium containing 2 ng/ml granulocyte macrophage colony-stimulating factor GM-CSF) obtained from Immunex, Seattle, WA and 4 ng/ml interleukin -4 (IL-4), obtained from Sigma. Cells are cultured for 5 days and then dendritic cells (DCs) harvested by centrifugation and used for experiments following verification by light microscopy examination and flow-cytometry.

DCs were infected with the virus at a multiplicity of infection (MOI) of 100.Infection experiment were carried out in polypropylene tubes to prevent the adherence of the cells. 50 µl of viral suspension were inoculated into 50 µl of cell suspension ($1.5x10^6$ cells) in complete RPMI-1640 medium containing 2% of autologous serum. After inoculation the cells were incubated 90 min at 37 C in 5% CO_2 at the complete RPMI-1640 medium containing 2% of autologous serum, than washed three times and incubated in RPMI-1640 medium containing 10% of autologous serum for additional 24h at 37 C in 5% CO_2. Expression of PSMA was tested by immunoblotting. Efficiency of infection of DC by the adenovirus in our experiments was 20% i.e. 20% of the DC were infected by the recombinant adenovirus.

In additional experiments DCs were obtained from HLA-A2+ patients, infected with adenovirus, and cultured with autologous T cells in CM for 3 days at 37°C. T cells were harvested at the end of the incubation, CD8+ T cells purified by negative depletion with anti-CD4 antibodies and complement and their cytotoxicity tested. The CD8+ T cells that had been stimulated by autologous DC infected with Ad5-PSMA were cytotoxic against the prostate tumor cell line LNCaP.FGC (also of the HLA A2+ phenotype), but not against Jurkat (T leukemia) or U937 (myelomonocytic cell line) cells. In comparison, freshly separated T cells showed no cytotoxicity against any of the three cell lines.

In vivo experiments:

Patient Treatment with Plasmid or Adenovirus

Study design:

One group of seven patients received three injections of XC PSMA-DNA vaccine (XC PSMA-

CD86 plasmid) at the same dose (100 ug) at one-week intervals. Five patients (see table 1) received 10,000 IU Leukine (Immunex, Seattle, WA at the site of the plasmid application immediately or 24 and 48 hours after the immunization.

Additionally, two months later, these seven patients and a group of 2 new patients received three injections of a recombinant, replication-deficient adenoviral (Ad5-XC-PSMA) vaccine ($5x10^8$ PFUs per application) at one -week intervals.

Plasmid was injected intradermally between the first and second toe of the right leg or intramuscularly. The viral vaccine was administered intradermally in the navel area.

Constant monitoring of the clinical state and the vital signs was carried out for 2 hours after vaccination. If stable, the subject was allowed to leave the hospital. A brief follow-up visit occurred 24 (and 48 in the case of GM-CSF innoculation) hours later.

Inclusion criteria:

All patients signed an informed consent form before admission into the study. Data from monitoring visits were shared with the patients as the study proceeded, and the patients were reminded that hey were free to withdraw from participation at any time.

Only patients with advanced, hormone-resistant cancer or patients unable to find or administer hormone therapy were included into the study.

Patients with a history of another malignancy or with a serious active infection or with another illness were excluded from the study.

Monitoring Studies:

Standard laboratory tests included CBC, urinanalysis, liver enzymes, antinuclear antibodies, erythrocyte sedimentation rate, PSA. Each patient had a pelvic CAT scan, chest radiograph and a cardiograph on entry and on week 20 (week 10 for the 2 patients immunized with virus only). Safety was defined as lack of untoward clinical or laboratory events, with particular attention to local and systemic reactions, as well evidence of anti-nuclear antibody.

Additionally, analysis of CD/HLA DR^+; $CD4^+$; $CD8^+$; $CD3^-/CD16^+CD56^+$; $CD3^+$; $CD1^+$; $CD25^+$; $CD19^+$ cells as well as CD4/CD8 ratio prior and following immunotherapy was performed by flow cytometry.

Results

Characteristics of participants:

Nine men, ages between 49 and 69 with advanced adenocarcinoma of the prostate, were included in the study. Three patients had a radical prostatectomy, 2 were in preparation for surgery, three were inoperable and one was operable but had other contraindications for surgery treatment. Two of the patients died due to advanced cancer disease.

Safety monitoring results:

The immunizations were well tolerated. No changes in vital signs occurred following injections or on follow-up visits.

Patients who received intradermal immunizations with plasmid had a minor DTH-like reactions 24 hours following the third immunization. Patients NN 8 and 9 developed a DTH reaction 24 hours following each administration of the recombinant adenovirus. Patients NN 1 through 7 had no DTH-like reactions 24 hrs after the first immunization with the viral vector, but developed DTH after the second and third immunization. All DTH-like local reactions were mild and resolved within 72-hrs post immunization.

Patient N 4 had a vesicular rash after the last viral immunization which was located on the back and which resolved in the next two days with no treatment.

Patient N 7 had a papular urticaria-like rash with small petechiae at the center which developed 24 hrs after the last plasmid immunization and which disappeared after the discontinuation of the antibiotic therapy he was receiving.

No significant changes occurred in erythrocyte sedimentation rate, CBC, serum creatinine or other blood chemistries, or urinanalysis. Serum liver chemistry values remained within normal range in all subjects.

No significant changes in the analysis of CD/HLA DR^+; $CD4^+$; $CD8^+$; $CD3^-/CD16^+CD56^+$; $CD3^+$; $CD1^+$; $CD25^+$; $CD19^+$ cells as well as CD4/CD8 ratio prior and following immunotherapy were detected.

No subject developed abnormal vital signs following injection, no significant increase in antinuclear antibodies titer were observed, and anti-DNA antibody was not detected.

For PSA values, CAT-scans, bone scintigraphy or lymph node metastases before and after immunization see tables 1 and 2.

Tables 1 and 2 show that in some patients the progression of metastatic prostate cancer was retarded or stopped.

Table 1. Patients were immunized initially three times at weekly intervals with PSMA plasmid. Two months later all patients but patient # 7 received three additional immunizations at weekly intervals with the recombinant adenovirus.

Patient #	Stage of disease	Type of immunization	Additional treatment	PSA(ng/ml) before	PSA(ng/ml) after	CT scan before	CT scan after	LN before	LN after	Bone metastases before	Bone metastases after	Side effects
1	$T_4 N_x M_2$ inopcrable	3x plsmd i.d. 3 x Ad5PSMA	orchiectomy Casodex	6.3	-	+++	+++	-	+	++	++	exitus
2	$T_2N_oM_o$ operable#	3x plsmd i.m. +GM-CSF 3 x Ad5PSMA	orchiectomy Androcur	14.38	0.28	+++	*	-	-	-	-	none
3	$T_4N_xM_o$ inoperable	3x plsmd i.d. 3 x Ad5PSMA	orchiectomy	33.0	0.04	+++	*	-	-	-	+	none
4	$T_4M_xM_2$ post BPH and TUR inoperable	3x plsmd i.d. +GM-CSF 3 x Ad5PSMA	orchiectomy (recently Flucinome)	1.11	3.8	++	++	-	-	+	++	none
5	$T_{2-3}N_oM_o$ in preparation for surgery	3x plsmd i.d +GM-CSF 3 x Ad5PSMA	MAB	3.01	0.05	++	*	-	-	-	-	none
6	$T_{3-4}N_xM_x$ post TUR	3x plsmd i.d. +GM-CSF 3 x Ad5PSMA	orchiectomy MAB	1.6	0.04	+++	*	-	-	-	-	none
7	$T_4N_xM_2$ post radical prostatectomy metastases	3x plsmd i.m. +GM-CSF	MAB	100		+++	++**	-		+++		exitus skin rash ***

Legend:

++; +++ increase in the size of the prostate gland or presence of metastatic tumor post radical prostatectomy (patient # 7)

-;+ lack (-) or presence of bone metastases or lymph node engagement

* significant decrease in the size of the prostate gland.

** - Patient # 7. Lack of urine excretion from both urethers due to metastases prior to the immune therapy. Appearance of diuresis from the right kidney one month after the last immunization. Died due to mechanical illeus following blockade of the rectum and sigmoideum by metastases.

*** - Patient # 7 had a mild skin rash 24 hrs post the third plasmid application which disappeared after discontinuation of the concurrent antibiotic therapy.

- Patient # 2 could not have surgery due to cardiovascular complications.

MAB - maximum androgen blockade with Zoladex, Casodex or Flucinome

orchiectomy - always bilateral

Table 2. Patients who were immunized with recombinant adenovirus 3 times at weekly intervals.

Patient #	Stage of disease	Type of immunization	Additional treatment	PSA(ng/ml) before	PSA(ng/ml) after	CT scan before	CT scan after	LN before	LN after	Bone metastases	Bone metastases	Side effects
8	T_4N_2M post radical prostatectomy metastases	3xAd5PSMA	MAB	32	NA	+++	NA	+++	NA	-	NA	none
9	T_4NM_2 post radical prostatectomy metastases	3xAd5PSMA	MAB	4.47	NA	+++	NA	-	NA	+++	NA	none

-; ++; +++ lack (-) or presence of local tumor metastases, or lymph node engagement

MAB - maximum androgen blockade with Zoladex, Casodex or Flucinome

NA - not available

It will be apparent to those skilled in the art that the examples and embodiments described herein are by way of illustration and not of limitation, and that other examples may be used without departing from the spirit and scope of the present invention, as set forth in the claims.

CLAIMS

We claim:

1. The process of causing expression in a mammal or cells thereof of antigens which are functionally inactive without affecting the efficiency of transcription and translation of DNA, translation of RNA, or the generation of antigenic peptides, consisting of the step:

administering to the mammal or cells thereof of an effective amount of RNA, plasmid DNA, or viral vector comprising DNA or RNA encoding an altered antigen which is functionally inactive yet is unaltered with respect to the efficiency of transcription and translation of DNA, translation of RNA, or the generation of antigenic peptides.

2. The process of claim 1 wherein the altered antigen is a truncated antigen.

3. The process of claim 2 wherein the truncated antigen is the extracellular domain of an antigen.

4. The process of claim 3 wherein the antigen is human prostate specific membrane antigen.

5. RNA or plasmid DNA encoding an altered human cancer-associated antigen which is functionally inactive yet is unaltered with respect to the efficiency of transcription and translation of DNA, translation of RNA, or the generation of antigenic peptides.

6. RNA or plasmid DNA of claim 5 wherein the altered human cancer-associated antigen is the extracellular portion of human prostate specific membrane antigen.

7. Plasmid DNA of claim 6 with ATCC Accession No. 203168.

8. The nucleic acid having the sequence of SEQ ID NO: 1.

9. The extracellular portion of human prostate specific membrane antigen encoded by the nucleic acid sequence of SEQ ID NO: 1.

10. Plasmid DNA of claim 5 wherein the extracellular portion of human prostate specific membrane antigen is encoded by the nucleic acid sequence of SEQ ID NO: 1.

11. A viral vector comprising RNA or DNA, said vector encoding an altered human cancer-associated antigen which is functionally inactive yet is unaltered with respect to the efficiency of transcription and translation of DNA, translation of RNA, or the generation of antigenic peptides.

12. A viral vector of claim 11 wherein the altered human cancer-associated antigen is the extracellular portion of the human prostate specific membrane antigen.

13. A viral vector of claim 12 wherein the viral vector is a recombinant replication-deficient adenovirus comprising DNA encoding an extracellular portion of the human prostate specific membranc antigen.

14. A viral vector of claim 13 having ATCC Acquisition No. _____.

15. A viral vector of claim 13 wherein the extracellular portion of the human prostate specific membrane antigen is encoded by the nucleic acid sequence of SEQ ID NO: 1.

16. A method for treatment of cancer comprising administering to a cancer patient in need thereof an effective amount of the RNA or plasmid DNA of claim 5.

17. The method of claim 16 wherein the patient is a prostate cancer patient and the RNA or the plasmid DNA is that of claim 6

18. The method of claim 17 wherein the plasmid is that of claim 7.

19. The method of claim 17 wherein the plasmid DNA comprises that of SEQ ID NO. 1.

20. A method for treatment of cancer comprising administering to a cancer patient in need thereof an effective amount of a viral vector of claim 11.

21. The method of claim 20 wherein the patient is a prostate cancer patient and the viral vector is the viral vector of claim 13.

22. The method of claim 21 wherein the viral vector is that of claim 14.

23. The method of claim 13 wherein the DNA encoding an extracellular portion of the human prostate specific membrane antigen has the sequence of SEQ ID NO: 1.

24. The method for treatment of cancer of claim 16 further comprising the steps waiting an appropriate interval after the step of claim 16, and administering to the patient a booster dose of an effective amount of the viral vector of claim 11.

25. The method for treatment of prostate cancer of claim 17 further comprising the steps waiting an appropriate interval after the step of claim 17, and administering to the patient a booster dose of an effective amount of the viral vector of claim 13.

26. The method for treatment of prostate cancer of claim 25 wherein the plasmid DNA is that of claim 7 and the viral vector is that of claim 14.

27. The method for treatment of cancer of claim 20 further comprising waiting an appropriate interval after the step of claim 20, and administering to the patient a booster dose of an effective amount of the RNA or plasmid DNA of claim 5.

28. The method for treatment of prostate cancer of claim 21 further comprising the steps waiting an appropriate interval after the step of claim 21, and administering to the patient a booster dose of an effective amount of the RNA or plasmid DNA of claim 6.

29. The method for treatment of prostate cancer of claim 21 further comprising the step waiting an appropriate interval after the step of claim 21, and administering to the patient a booster dose of an effective amount of the RNA or plasmid DNA of claim 7.

30. A method for treatment of cancer comprising administering to a cancer patient in need thereof an effective amount of autologous dendritic cells that have been exposed in vitro to either or both the RNA or the plasmid DNA of claim 5, or the viral vector of claim 11.

31. The method for treatment of cancer of claim 30 wherein the patient is a prostate cancer patient, the RNA or the plasmid DNA is that of claim 6, and the viral vector is that of claim 12.

32. The method for treatment of prostate cancer of claim 31 wherein the RNA or plasmid DNA is that of claim 7 and the viral vector is that of claim 14.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/20508

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 15/63, 15/79, 15/11, 5/10, 15/09; A61K 48/00

US CL :435/ 325, 375, 320.1; 514/44; 424/93.21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S 435/ 325, 375, 320.1; 514/44; 424/93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CANCER LIT, EMBASE, CAPLUS
search terms: antigen, inactive, truncated, peptide, cancer, dendritic

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KNIGHT et al. Influence of Dendritic Cells on Tumor Growth. Proc. Natl. Acad. Sci. USA. July 1985, Vol. 82, pages 4495-4497, see entire article.	1-32
Y	KOKONTIS et al. Increased androgen receptor activity and altered c-*myc* expression in prostate cancer cells after long-term androgen deprivation. Cancer Research. 15 March 1994, Vol. 54, No. 6, pages 1566-1573, see entire article.	1-32
Y	SANTAMBROGIO et al. Altered peptide ligand modulation of experimental allergic encephalomyelitis: immune responses within the CNS. J. of Neuroimmunology. January 1998, Vol. 81, pages 1-13, see entire article.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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18 JANUARY 2000

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/20508

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to cla
Y	WO 92/09690 A2 (GENENTECH, INC.) 11 June 1992, see entire document.	1-32
	APOSTOLOPOULOS et al. The immunogenicity of MUC1 peptides and fusion protein. Cancer Letters. 23 March 1995, Vol. 90, No. 1, pages 21-26, see entire article.	1-32
Y	KIM et al. Altered expression of mucin genes and antigens in pancreatic cancer. Recent Adv. Gastroenterol. Carcinog. I, Int. Conf. Gastroenterol. 1996, Vol. 1, pages 187-197, see entire article.	1-32
Y	CERIANI et al. Epitope Expression on the Breast Epithelial Mucin. Breast Cancer Research and Treatment. 1992, Vol. 24, pages 103-113, see entire article.	1-32
Y	WO 94/28113 A1 (BIOTOP S.A.S. DI RITA CASSARIN) 08 DECEMBER 1994, see entire document.	1-32
Y	WO 93/20185 A1 (STEINMAN et al.) 14 October 1993, see entire document.	1-32
Y	LEWIS et al. Definition of tumor antigens suitable for vaccine construction. Seminars in Cancer Biology. December 1995, Vol. 6, pages 321-327, see entire article.	1-32
Y	NGUYEN et al. Membrane-bound (MUC1) and secretory (MUC2, MUC3, and MUC4) mucin gene expression in human lung cancer. Tumor Biology. 1996, Vol. 17, No. 3, pages 176-192, see entire document.	1-32